

## MICROBIAL PERSISTENCE\*

### I. THE CAPACITY OF TUBERCLE BACILLI TO SURVIVE STERILIZATION IN MOUSE TISSUES

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Microbial persistence is the term used to signify the phenomenon whereby microbes that are susceptible to a drug *in vitro* can nevertheless survive long continued exposure to it *in vivo* (1, 2).

An unusual form of microbial persistence displayed by tubercle bacilli of human origin was described in previous reports from this laboratory (3-5). Relatively large populations of bacilli (one to three million cells) could be made to "vanish" uniformly from the tissues of mice that had received two compounds; the nicotinamide derivative pyrazinamide, and isoniazid, in appropriate time-dose relationships. The criteria for "vanishing" were failure to detect any bacilli by; microscopy, culture, or guinea pig inoculation of the tissues of the infected mice. The "vanishing" did not mean that the bacilli were totally eliminated from the tissues. On the contrary, after a 90 day treatment-free interval, culturable tubercle bacilli could be demonstrated in approximately one-third of the animals. The question immediately arose whether all of the tubercle bacilli had been eradicated in the other two-thirds of the animals in each experiment, or whether the bacilli were still present in them but were in a latent state.

By latent is meant a phase during which the infection is hidden beyond all diagnostic reach. In such a phase, the bacilli conceivably could be present in some altered form or they could be unaltered, but so few in number that they escape detection. In view of the demonstrated sensitivity of the culture techniques employed, it seemed clear that at the most only a very few *unaltered* bacilli could be present and escape detection. It was noted (4), however, that if a microbial alteration took the form of sterilization without death, fairly large populations, being nonculturable, might vanish from the tissues because of the insensitivity of microscopy as a detection technique. In the studies reported (3), acid-fast bacilli could not be found with any regularity on microscopy of tissue sections when the organ census was below  $1 \times 10^6$  per ml and only

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a rare tubercle bacillus could be found in sections from an organ with a microbial census as low as  $1 \times 10^4$  per ml.

Accordingly, a series of studies has been made of the vanishing phenomenon to determine whether the tubercle bacilli are wholly eradicated from that portion (the majority) of the animals in which they fail to reappear; or whether instead they are uniformly sterilized or rendered latent in the tissue.

In this report, evidence is presented that when the tubercle bacilli vanish from groups of animals, the bacilli are not eradicated, but are in a sterile<sup>1</sup> state that can persist for periods ranging from a few months to more than a year. In an accompanying paper (6), observations are presented that serve to characterize this phenomenon of the sterilization of tubercle bacilli *in vivo*.

#### *Materials and Methods*

For these studies, the "vanishing" of large populations of tubercle bacilli from the lungs and spleens of groups of mice was produced as previously described (3). In its essentials, the procedure consisted of infecting large numbers of mice (male albino, Webster Swiss strain) intravenously with 0.2 ml of a  $10^{-1}$  dilution of a 7 day culture of *Mycobacterium tuberculosis* (H37Rv) within 20 min of the start of a 12 wk course of drug administration. Isoniazid,<sup>2</sup> 0.0125%, and pyrazinamide,<sup>3</sup> 2.0%, were administered together in the daily diet mixed with finely ground food pellets.

At appropriate intervals, 10 to 15 mice were removed from each experimental group and killed. The lungs and spleens were homogenized and cultured by quantitative techniques originally described by Fenner, Martin, and Pierce (7), and described in detail in a preceding paper (3).

In a total 12 yr experience with this model (present studies included), a complete disappearance of culturable tubercle bacilli from all animals sacrificed at the 12 wk point has failed to occur in 3 of 33 individual experiments. These three exceptions consisted of the recovery of culturable bacilli from 2 of 10 mice in one experiment in 1955 and from one of 15 and one of 16 animals respectively in two experiments in 1961. As discussed in the following paper (6), the "sterilization failures" involving the 2 animals in 1961 were questionable while the failure in 1955 seems unquestionable.

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<sup>1</sup> In this paper and the one that follows, the words *sterile* and *sterilization* refer only to the tubercle bacilli and are used in the biologic sense of "not capable of reproduction". Such a state may or may not be reversible. All available methods for the detection of living microbes in the tissues are based on their capacity to multiply. Once this is blocked, it obviously becomes impossible to determine directly at a particular point in time whether living microbes are present or have been exterminated. The wide interest today in microbial plasticity coupled with our quite limited technics for direct microbial observations are making it necessary to impose such restrictions on familiar words when dealing with concepts like the viability or the nonviability of microbes. The justification for using the word *sterile* in so restricted a fashion, therefore, is that only by so doing, can we describe with precision what has actually been observed and the limits of what is presently demonstrable.

<sup>2</sup> Isoniazid supplied by E. R. Squibb & Sons, Division of Olin Mathieson Chemical Corp., New York.

<sup>3</sup> Pyrazinamide supplied by Merck Sharp & Dohme, West Point, Pennsylvania.

## OBSERVATIONS

*Attempts to Increase Revival*

In the model employed, microbial revival 12 wk after the "vanishing" had been demonstrated, occurred in slightly more than one-third of the animals subjected to the procedure (3, 4). A question for study, therefore, was whether in the other two-thirds of the animals, the populations of tubercle bacilli had been eradicated. The evidence of microbial revival was the demonstrated presence in the tissues of tubercle bacilli capable of multiplication on artificial media. Evidence for the occurrence of eradication had to be studied indirectly, by attempts to effect a substantial increase in the incidence of microbial revival.

*Low Dosage Cortisone and other Procedures.*—A series of procedures that had been observed or reported (8, 9) to have favored the growth of tubercle bacilli in laboratory animals were employed, usually for a 1 month period, within the first 3 months of the posttreatment period. The procedures included: intraperitoneal injection of large quantities of heat-killed BCG; periodic deprivation of food; exposure to ionizing radiation; the intramuscular administration of suramin (8); infection with influenza A<sub>2</sub> (strain Japanese 305, unadapted to mice); the daily subcutaneous administration of 20% alcohol; and the administration of cortisone 0.1 mg each day for 4 wk during the 1st, 2nd, or 3rd month of the standard drug-free interval. This daily dosage of cortisone had been previously shown to produce a substantial increase in the census of tubercle bacilli in the mouse tissues (10). All of these procedures, including the cortisone administration, failed to increase the incidence of microbial revival in the 12 wk post-treatment period.

*High Dosage Cortisone.*—The effect of a tenfold increase in cortisone dosage was then studied. On this dosage, 1.0 mg daily, the animals lost weight, appeared sick, and some died. Consequently, the question had to be studied in four consecutive experiments in which the daily cortisone administration was limited to a 20 to 28 day period in one of the first 4 posttreatment months. In each experiment, the presence of culturable tubercle bacilli was determined in the animals that had received cortisone for at least 20 days (Table I, Fig. 1).

When the cortisone was given in the 1st posttreatment month there was no microbial revival in either the 19 mice that had received daily cortisone or in the 15 similarly prepared mice that had received no cortisone. In the 2nd month, there were 4 instances of revival in the 13-animal cortisone group and there was no revival in the 15-animal control group. In the 3rd and 4th months, however, tubercle bacilli were cultured from all 26 animals<sup>4</sup> that received cortisone and from 19 of the 30 control animals. These data for the 3rd and

<sup>4</sup> In 2 of the 17 animals that received 20 days of cortisone in the 3rd posttreatment month, and in 6 of the 17 in the 4th, the homogenate cultures were so overgrown with a variety of bacteria that determination of the presence or absence of tubercle bacilli was not possible.

4th months are arranged in fourfold tables in Tables II and III. In Table II, the data for microbial revival in the animals that received cortisone are compared with the revival data for the concurrent control animals. In Table III, the basis for comparison is the revival data for the 3rd posttreatment month from a total of 16 individual experiments in which no cortisone was given and the 3rd posttreatment month was studied. By either method of comparison there is a significant difference, in terms of microbial revival, between the animals that received cortisone and those that did not ( $P < 0.01$  concurrent controls;  $P < 0.001$  "3rd month" controls). On probit analysis, the time required for microbial revival to occur in the spleens of one-half the animals, the  $RT_{50}$  was found to be 2.2 months for the cortisone animals with a 95% fiducial interval of 2.1 to 2.4 months. This  $RT_{50}$  of 2.2 months is to be compared

TABLE I

*Influence of Cortisone on Sterile Tuberculous Infections in Mice Induced by Pyrazinamide-Isoniazid Therapy*

Number of mice from which tubercle bacilli were cultured after at least 20 days of cortisone, or no cortisone, during the first 4 months following 12 wk of antimicrobial treatment.

Month	No. of mice			
	Cortisone		No cortisone	
	Cult. pos.	Cult. neg.	Cult. pos.	Cult. neg.
1	0	19	0	15
2	4	9	0	15
3	15	0	10	5
4	11	0	9	6

with an  $RT_{50}$  of 7 months found for the total experience in the absence of cortisone as set forth immediately below.

#### *The Course of Natural Revival*

A series of long term experiments were performed to determine the incidence of microbial revival under "natural" conditions for comparison with the high incidence of revival evoked by the 1.0 mg daily dosage of cortisone administered within the 3rd or 4th posttreatment month.

In one such experiment, 137 infected mice received the standard drug-regimen for the 12 wk period. At this point, 77 mice were sacrificed and their tissues were cultured for tubercle bacilli by a variety of procedures set forth in detail in a subsequent section of this paper. No tubercle bacilli were cultured. The remaining 60 mice were maintained without drug treatment and sample groups of 15 animals each, were sacrificed at 3, 6, 9, and 12 months after the com-

pletion of drug administration. The incidence of microbial revival at these observation points may be seen in Fig. 2. In addition, the natural microbial revival in the posttreatment period as a composite experience of 22 experiments is presented in Fig. 3. As may be seen in the spleens or the lungs, the percentage of mice with culturable tubercle bacilli rose from zero at the end of the 1st treatment-free month, to a maximum of 70 at the end of the 9th posttreatment month. 3 months later, at the end of a year without treatment, tubercle bacilli

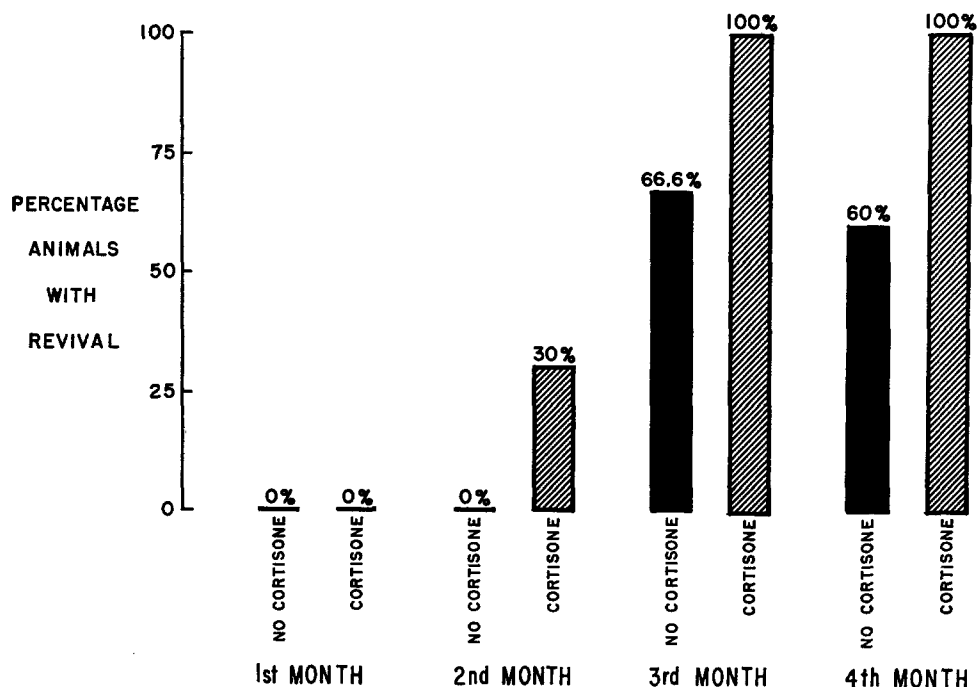


FIG. 1. Cortisone effect on per cent of mice with culturable tubercle bacilli in spleens or lungs in first 4 months following induction of sterile state. Cortisone dosage 1.0 mg daily for at least 20 days in the single month so indicated.

were cultured from the spleens of 33 of 57 animals, and from the lungs of 21 of 57 animals.

Both the composite data on microbial revival and those from the representative experiment appeared to follow a normal sigmoid curve. When subjected to probit analysis, the composite data for both lungs and spleen closely adhered to a straight line (Fig 4). This confirms the normal character of the revival curves and thus suggests that microbial revival is reproducible for the average animal. From the observed data, the  $RT_{50}$  for splenic populations of

tubercle bacilli was 7 months. For the lungs, the  $RT_{50}$  was not reached in the observation period of 1 yr.

Microbial revival thus appeared to occur naturally in approximately two-thirds of the animals by the end of the 9th posttreatment month and could be evoked earlier in a considerably higher percentage of animals by the administration of near lethal doses of cortisone. It appeared, therefore, that living tubercle bacilli were present in most, if not all, of the animals in the standard

TABLE II

*Effect of Cortisone on Revival of Sterilized Populations of Tubercle Bacilli*

Number of cortisone-treated or control mice from which tubercle bacilli were cultured. Cortisone administered for at least 20 days within either the 3rd or the 4th month after drug-induced sterilization.

	Animals		Totals
	Cult. pos.	Cult. neg.	
Cortisone	26	0	26
No cortisone	19	11	30
Totals.....	45	11	56

TABLE III

*Effect of Cortisone on Revival of Sterilized Populations of Tubercle Bacilli but Limited to 3rd Post Therapy Month and Including Comparable Data on "No Cortisone" Groups from 16 Other Standard Experiments*

	Animals		Totals
	Cult. pos.	Cult. neg.	
Cortisone	15	0	15
No cortisone	66	153	219
Total.....	81	153	234

model at the completion of 12 wk of the two drug administration despite the uniform nonculturability of the bacilli at that point. Accordingly, an intensified effort was made to detect the bacilli during this phase in which they had vanished.

*Culture or Animal Passage as Detection Methods*

In the experiment on natural microbial revival described above (Fig. 2), a separate sample of 77 mice was sacrificed 48 hr after the completion of the

12 wk period of drug administration, and the spleen and lungs<sup>5</sup> were studied for the presence of tubercle bacilli. Subgroups of the 77 mice were examined by: microscopic examination of stained sections of lung and spleen; culture in various tissue dilutions on appropriate media; splenic explants and primary cell cultures and splenic implantations on normal mouse kidney cells; entire organ homogenates cultured in thin layers on 105 cm<sup>2</sup> culture plates; and guinea pig

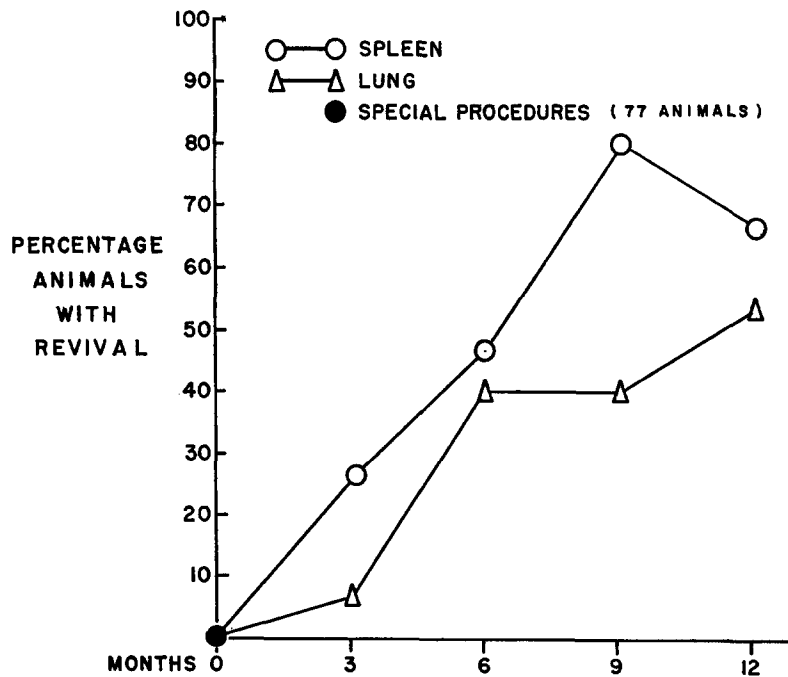


FIG. 2. Percentage of mice with culturable tubercle bacilli in spleens and lungs in 1 yr period starting immediately after completion of 12 wk standard treatment regimen of pyrazinamide and isoniazid. Sterilization at end of treatment demonstrated by failure to detect living bacilli in a 77 animal sample studied by a variety of procedures.

inoculation with subsequent tuberculin testing and autopsy of the recipient animals.

The mice had been originally infected with approximately three million tubercle bacilli. Without exception, these attempts to demonstrate tubercle bacilli were unsuccessful; accordingly, the observations are presented in condensed form as follows.

<sup>5</sup> In previous studies (1, 2) and in experiments cited below, it has been observed that when tubercle bacilli are not demonstrable in the spleen or the lungs, they are not demonstrable in the liver, kidneys, or lymph nodes.

*Massive Culture of Homogenate on Oleic Acid Albumin Agar Plates*—The lungs and spleens of 15 mice killed during the 1st month after the 12 wk treatment period ended were homogenized separately in 2.5 ml of 2.0% bovine albumin and 0.5 ml of this homogenate was inoculated on each of three agar plates which were incubated for a minimum of 4 wk.

*Spleen Cultures After Double Washing and Centrifugation*.—The spleens from 10 mice killed 11 days after completion of drug administration were each homogenized in 10 ml of 0.1% albumin in normal saline and immediately thereafter another 10 ml of diluent were added. After centrifuging this for 30 min at 3000 RPM, the supernatant was removed, 10 ml diluent were added and the centrifuging was repeated. The deposit from each of the 10 tubes was

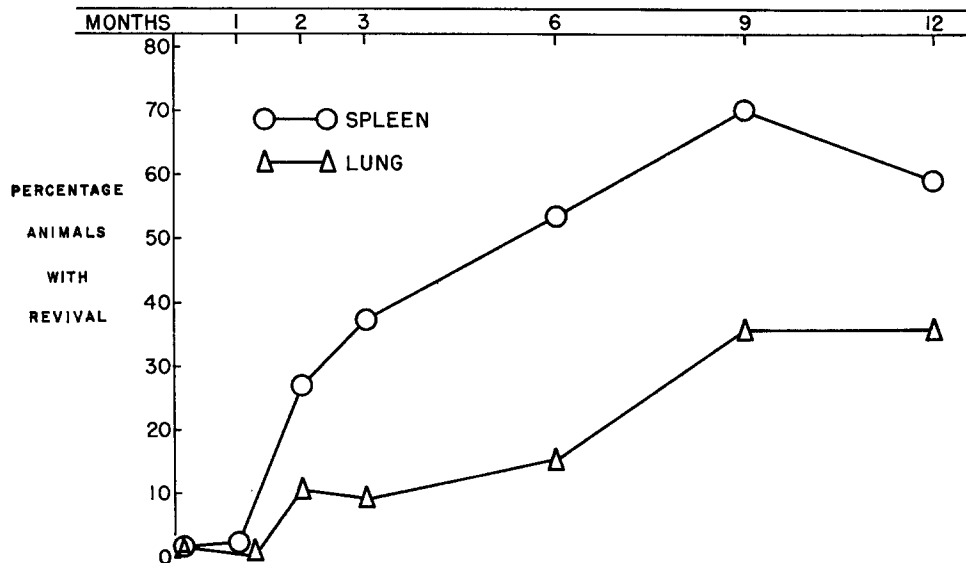


FIG. 3. Per cent of mice with culturable tubercle bacilli in spleens and lungs at intervals in the year following 12 wk of the standard regimen of pyrazinamide and isoniazid started at the time of inoculation. Composite of 22 experiments.

then resuspended in 2.0 ml of the diluent and distributed on four oleic acid albumin agar plates.

*Cultures of Trypsinized Spleen Homogenates*.—The spleens from 10 mice killed 10 days after the completion of drug administration were minced, and transferred to a grinding tube containing 2.0 ml of 0.25% trypsin in buffered saline. After incubation at 37°C for 20 min, 3.0 ml of 0.1% albumin-saline were added, the tissue was ground and spread on two plates of Dubos medium and two plates of Dubos medium with 0.1% charcoal (Norit A).

*Splenic Tissue Culture*.—The spleens from 20 mice were used for attempts to isolate tubercle bacilli by growth in tissue culture. Ten mice were killed 3 days after cessation of drug administration and the spleens were homogenized. One-fourth of the homogenate from each animal was used for direct tissue culture in 10% horse serum, 90% Eagle's solution (11) containing 200 µg/ml penicillin. Smears were made 3, 4, 5, and 6 wk later and stained for acid-fast bacilli. No bacilli were detected by microscopy.

Subcultures were made at the same time on oleic acid albumin agar.



The remaining three-fourths of the homogenate from each mouse was used to inoculate normal mouse kidney tissue cultures in a medium of 10% horse serum and 90% Eagle's solution containing 200  $\mu$ g of penicillin per ml. Two cultures each were set up in regular screw cap tubes and four in Leighton tubes. Slides were smeared at 3 and 4 wk after inoculation and stained for acid-fast bacilli. No bacilli were detected by microscopy.

Two oleic acid albumin agar plates were also inoculated from each of these homogenates.

The other 10 mice were sacrificed 4 days after cessation of treatment. After removal, iris scissors were employed to mince the tissue into 1 mm pieces, and these from each spleen were placed on 11 or 12 special microslides. Chicken plasma was dropped on spleen bits and a clot

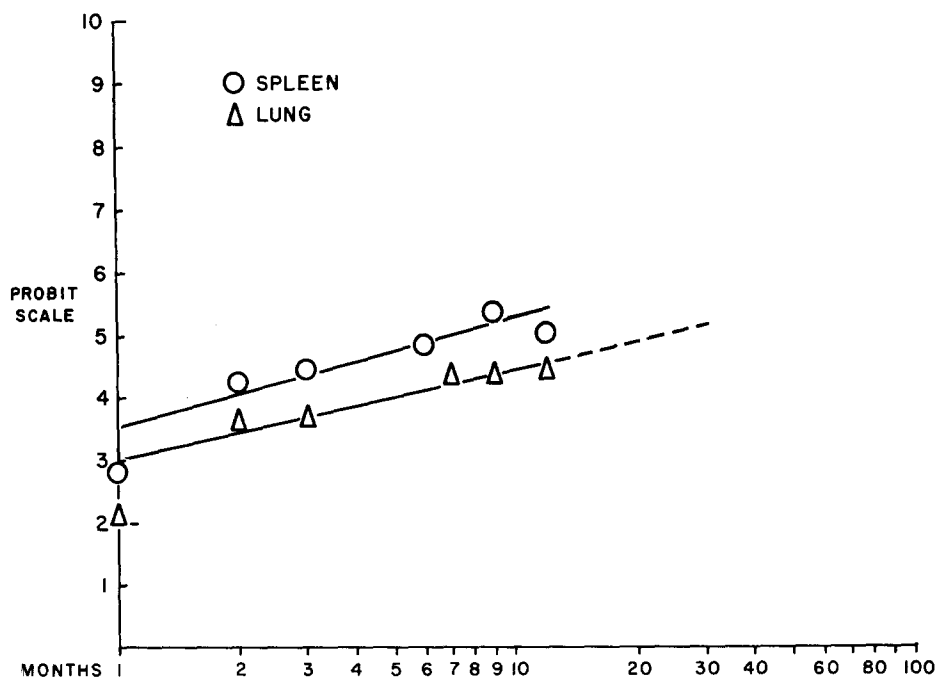


FIG. 4. Per cent of mice with culturable tubercle bacilli in spleens and lungs at intervals in the year following 12 wk of the standard regimen of pyrazinamide and isoniazid started at the time of inoculation. Probit analysis of combined data from 13 experiments.

formed. The microslides were placed in screw-capped tubes. Eagle's medium with 10% heat-inactivated horse serum was added and it was incubated in 5%  $\text{CO}_2$ . Medium was replaced every 3 to 4 days during the initial 2 wk, and weekly thereafter. The clots were trimmed periodically. Three distinct tissue cell types were seen. After 35 days of observing, including the weekly staining of one of the microslides from each of the 10 animals, no tubercle bacilli were detected.

*Guinea Pig Inoculation with Lyophilized Spleen Homogenate in Mineral Oil.*—The spleens from 12 mice killed 4, 5, and 6 days following the end of a standard 12 wk course of treatment were ground separately in 2.0 ml of 0.1% albumin-saline. From each homogenate, 0.5 ml was used to inoculate an oleic acid albumin agar plate with 5 separate drops and the remainder

was lyophilized before suspending in 1.5 ml liquid petrolate. These suspensions were inoculated subcutaneously into 12 guinea pigs. At 3, 4, 5, 6, and 8 wk after inoculation, groups consisting of 3 inoculated animals and 1 uninoculated animal were tested intradermally for reaction to 0.2 ml of 1:10 old tuberculin (Eli Lilly and Company, Indianapolis). No positive reactions developed. The 11 guinea pigs still alive were autopsied 98 days after inoculation and no evidence of tuberculous disease was found.

*Culture of Total Spleen Homogenate on a Large Surface of Agar.*—Ten mice were killed 10 days after the completion of drug administration and the spleen of each was homogenized in 10 ml albumin-saline diluent. The homogenates were pooled with an additional 10 ml of diluent and the total was spread over an enriched agar surface on the side of four Blake bottles. Each bottle provided an agar surface of approximately 105 cm<sup>2</sup>.

The above studies were all conducted on the tissues of 77 animals from a single experiment, the later course of which is portrayed in Fig. 2. In a number of other experiments, additional searches for the presence of culturable tubercle bacilli in mice that had received the two drugs in the standard model were made as follows:

*Slide Cultures of Splenic Touch Preparations and of Splenic Homogenates.*—3 days after the completion of the standard 12 week two drug regimen, 20 mice were sacrificed. The spleens of 10 animals were homogenized in 1.0 ml of 3% albumin-saline. The spleens of the other 10 mice were cut across the mid portion with a scalpel blade under aseptic conditions and the cut surface was pressed several times at different positions on the special microslides. The homogenates were similarly prepared on microslides; appropriate controls from the spleens of animals infected 24 hr previously with the standard inoculum of tubercle bacilli were prepared and all slides were cultured in liquid oleic acid albumin medium for 21 days. In general, the techniques employed were those reported by Noufflard and Berteaux (12), and by Pierce (13).

*Blind Transfer of Splenic Homogenates to Normal Mice.*—The individual spleen and lung homogenates from 21 mice at the 12 wk treatment point were inoculated in doses of 0.5 ml intraperitoneally into 41 normal mice. The "normal mice" were sacrificed in groups of 5 or 6 or 10 at 1, 2, 3, 4, 5, 6, and 7 months after inoculation and their lung and spleen homogenates planted on oleic acid albumin agar plates.

*Transplant of Whole or Diced Spleen beneath Muscle Layers of Guinea Pig Abdominal Wall.*—3 days after cessation of therapy, 10 mice were sacrificed and their spleens were removed. The intact spleens of 5 mice were transplanted beneath the abdominal wall muscle of 5 guinea pigs. The spleens from the other 5 mice were cut into pieces with the aid of a scalpel. These pieces of spleen were placed beneath muscle layers of the abdominal wall and the flank of 5 guinea pigs.

At 3, 4, 5, 6, and 8 wk after the transplants of intact spleen or splenic pieces, 1 guinea pig from each group and 1 normal guinea pig were tested intradermally for reaction to 0.2 ml of 1:10 old tuberculin (Lilly). No positive reaction was observed in any of the 3 test animals or controls from either group. There was no evidence of disease seen in tissues of the 8 remaining guinea pigs 112 days after insertion of the whole or divided spleen.

*Culture of Liver, Kidney, Lymph Nodes.*—The lungs, spleens, livers, kidneys, and the inguinal axillary, cervical and tracheobronchial lymph nodes from 10 mice were homogenized, examined by microscopy, and cultured by the more sensitive of the two techniques described in a previous report (3).

*Entire Mouse Ground and Cultured.*—Five mice, from one experiment, were sacrificed 5 days after completion of the 12 weeks of two drug therapy. Separate homogenates were made of the lungs, spleen, kidneys, liver, brain, lymph nodes, and digestive tract. Bone and flesh parts were

ground in a special Waring grinder. The homogenates were cultured on the surface of solid oleic-acid albumin agar.

All of the above attempts to increase the sensitivity of the culture or animal inoculation techniques for detection of tubercle bacilli in the mouse tissues were unsuccessful. At the time the bacilli had vanished, it was not possible in any case to detect their presence by procedures based on their capacity to multiply in vivo or in vitro. Accordingly, the sensitivity of the techniques for detecting nonmultiplying bacilli in the tissues by staining and microscopy was examined. In the course of the preceding experiments, examination of appropriately stained multiple sections of spleen and lungs and of their homogenates had failed to reveal acid-fast bacilli. Nevertheless, it seemed advisable to make a systematic and more intensified effort to increase the sensitivity of these techniques.

#### *Microscopy as a Detection Method*

In these microscopy studies, tissue sections were not subjected to examination. For, in the preceding experiments and in previously reported studies with other antimicrobial drugs (3), a prolonged search of sections had shown that only a rare bacillus could be found when the culturable populations in an organ were as low as  $1 \times 10^4$ . Moreover, in the previously reported studies of the "vanishing phenomenon" (4) stained smears of the homogenized organs had been examined by microscopy and no acid-fast bacilli had been detected. The examinations were of the type made on sputum smears in a clinical laboratory, however, and no attempt was made to examine the entire smear intensively. Accordingly, an intensified microscopic examination of the homogenized tissue was instituted.

*Intensified Microscopic Examination of Homogenates of Spleen and Lung.*—By the standardized procedures outlined above, a group of 165 mice was infected with tubercle bacilli and divided into an untreated control group and a group that received pyrazinamide and isoniazid for a 12 wk period. On sacrifice, the lungs and spleen of each animal were homogenized and the major portion of the homogenate was cultured for tubercle bacilli.

Microscopic examination of the homogenate of lung and spleen of the control animals was made at 12 and 33 days following infection with tubercle bacilli. The homogenates from the drug-treated animals were studied at the completion of the 12 wk period and at 49, 64, and 92 days thereafter. The homogenates of lung and spleen were made in 2.5 ml of diluent from which 0.02 or 0.01 ml drops were spread in small circles (1.0 to 2.0 cm diameter) on two slides. One slide was stained by the Ziehl-Nielsen method and the other with the fluorescent dyes auramine and rhodamine.

Under the oil immersion objective, the smears were examined microscopically by scanning as completely as possible with the mechanical stage. The searches were intensive. In the cases in which complete counts were made 4 to 6 hr were devoted to the examination of each slide and the number of microscopic fields searched per smear approximated 7000.

In the first experiment, in 15 of 21 animals examined after the 12 wk period of drug administration, acid-fast bacilli morphologically like tubercle bacilli

were found in the homogenate smears. The total number of these microbial forms per smear ranged from one to six; the usual number was two or three detected in a 4 to 6 hr search. The bacilli were seen in the splenic homogenates from 14 of 21 animals and in the pulmonary homogenates of 6 animals. In 5 instances the acid-fast bacilli were detected in homogenates of both organs.

This experiment was repeated because it turned out to be one of the 2 experiments (see Materials and Methods) in which the uniformity of the vanishing of the tubercle bacilli at the 12 wk point had to be classified as questionable.<sup>6</sup> The design of the second experiment was identical to the first and no tubercle bacilli could be cultured from the lungs or spleen of any of the animals sacrificed at the 12 wk point.

In this second experiment, microscopic examination revealed acid-fast bacilli in the homogenate smears from 15 of the 15 animals. In 15 cases the bacilli were found in the splenic homogenates; in all 10 pulmonary homogenates examined, they were also detected. The number of bacilli per smear was considerably higher than in the first experiment, ranging up to 300 in spleen and 15 in lungs. In these two, and a third similar experiment, the smeared homogenates from 56 animals were examined at the 12 wk point when all tubercle bacilli had vanished as determined by culture or animal transfer. The microbial forms, indistinguishable from tubercle bacilli, were detected in the specimens from all 56 mice. As the microbial forms were detectable by either staining procedure, no systematic comparison was made of fluorescence microscopy and ordinary light microscopy in terms of efficiency of detection. The definite impression was gained, however, that the microbial forms were more readily detected by the fluorescence procedure and thus that the total counts were higher when obtained by this method.

*Microscopy of Tissue from Normal Mice.*—As the numbers of bacillary forms detected were relatively small and similar numbers of acid-fast bacilli have been noted in the tissues of normal guinea pigs (14), the lungs and spleens of 7 normal mice were studied by the same techniques as were used in the previous experiments. No acid-fast bacilli or fluorescent microbial forms were found in the homogenate smears from any of the 7 animals.

*Addition of Known Numbers of Culturable Units of Tubercle Bacilli to Homogenate of Lung and Spleen.*—To obtain an approximation of the smallest census of *M. tuberculosis* detectable by the intensified microscopic search of spleen or lung homogenate, the following experiment was performed:

A 7 day culture of *M. tuberculosis* strain H37Rv on Tween albumin medium was diluted and plated out on oleic-acid albumin agar to determine the number of culturable units per ml. As

<sup>6</sup> As discussed in the accompanying paper (6) it is considered highly unlikely that this experiment represented a failure to attain the uniform vanishing of the bacilli. Of the 15 animals sacrificed at the 12 wk point, the splenic homogenate of one yielded a large single colony of acid-fast bacilli that failed to produce niacin, and grew rapidly at room temperature.

the colonies could not be counted immediately, a temporary estimate of the expected result was used to make up dilutions for addition to organ homogenates.

Separate homogenates of the lungs and spleen from 7 normal 20 g mice were made up to 3.0 ml volume each, and amounts of the bacillary suspensions were added and well mixed to give estimated final numbers of culturable units of 20,000; 10,000; 5000; 1000; 500; 100; and 50 per ml of homogenate.

*Clumping of Bacilli.*—In preliminary experiments, considerable difficulty was experienced because of clumping of the bacilli. Even a few bacilli aggregated as a clump appeared to have a considerably higher degree of visibility in the artificially seeded homogenate smears than the one to four individual acid-fast bacilli that represented the usual finding in the smears from the infected animals. Consequently, the microbial suspension was prepared by the filtration of a 7 day culture through two layers of No. 42 Whatman filter paper. When the filtered culture was

TABLE IV  
*Fluorescence Microscopy of 0.01 ml Smears After Addition of Tubercle Bacilli to Splenic Homogenates: Stain, Auramine-Rhodamine*

Tubercle bacilli added as calculated from original culture plate count	Tubercle bacilli added as calculated from plate counts of homogenates	Microbial forms detected
<i>Spleen</i>		
339	565	365
78	99	115
9	11	4
<i>Lung</i>		
12	14	22
1	1	9
<0.5	<0.5	7
<0.5	<0.5	4
<0.5	<0.5	2

used, the acid-fast bacilli detected in the smears of the homogenate suspension were almost all present as single cells.

The number of bacilli actually present was calculated from the plate counts made on the original culture after it had been filtered. Slides and plate counts of these artificially seeded homogenates were prepared and examined as in the previous experiments. Only the auramine and rhodamine dyes and fluorescent microscopy were used in this experiment.

As may be seen in Table IV, the number of added units detected ranged from 2 to 365 per 0.01 ml of lung or splenic homogenates. By using fluorescence microscopy and by pushing the microscopic examination of a smear to an extreme not ordinarily practicable, there was not too great a variance and it was possible to identify most of the microbial forms in a smear. Using these data, crude estimates were made with the assumption that the microbial forms detected by microscopy represented the total number of living tubercle bacilli present. By this crude calculation (which ignores possible quantitative differ-

ences between the two forms of microscopy) the populations of tubercle bacilli when "vanished" would range, in general, from one to several thousand cells per ml of homogenized tissue. This would represent less than 1% of the population density 24 hr after infection. In one of the three experiments, however, the estimated census in several animals was higher with a maximal estimated

TABLE V

*Estimated Census of Sterile Populations in Spleens on Assumption that Visible Microbial Forms are the Sterile Bacilli*

Per cent of census at 24 hr. Representative counts from 48 animals.

24 hr census per ml Homogenized spleen	Pyrazinamide and isoniazid administration	Mouse	Stain	Smear count per ml (estimate)	Per cent of 24 hr census
7,203,000	12 wk	1	ZN	1,770	0.025
		2	"	4,425	0.061
		3	"	21,240	0.29
2,691,000	12 wk	1	"	1,050	0.04
		2	"	2,100	0.08
		3	"	3,150	0.12
		4	"	6,300	0.23
10,140,500	12 wk	1	"	69,030	0.68
		1	AR	101,000	1.0
		2	AR	208,860	2.0
		3	AR	538,080	5.3
3,136,500	26 wk	1	AR	50,625	1.6
		2	AR	35,100	1.1
		3	ZN	7,965	0.25
3,875,900	26 wk*	1	ZN	2,200	0.06
		2	AR	25,296	0.65
10,196,666 (2 weeks)	37 wk	1	AR	30,090	0.297

ZN, Ziehl-Nielsen; AR, auramine-rhodamine.

\* Counts made after 8 wk cortisone treatment when cultures were negative.

population density of 2 and of 5% of that present 24 hr after infection. Representative examples of these calculations are presented in Table V which also includes the findings on microscopy of tissues from 6 animals that had received the pyrazinamide and the isoniazid for considerably longer periods. A detailed presentation of the last named experiments may be found in the accompanying paper (6).

*Influence of Acquired Resistance on Microbial Revival*

If all of the visible forms represented revivable tubercle bacilli, the populations that persisted in a nonculturable state after 12 wk of treatment as determined by 42 smear counts would represent at the most, only about 5% and usually less than 1% of the total number of tubercle bacilli present in the spleens or lungs.

Although the census of tubercle bacilli when vanished is presumably low, the census during the first 24 hr after infection in the standard model was high. In a total of 21 experiments the splenic census ranged from log 4.93 to log 6.94 with a median of log 6.32 per ml of homogenate. Populations of this size would be expected to call forth an immune reaction in the host. This acquired resistance would be expected to endure well past the 12 wk of drug administration and hence might interfere with the detection of the much smaller microbial populations presumably present at the beginning of the posttreatment period. The persisting tubercle bacilli might fail to multiply freely, not because they were in an altered state, but because they were present in only small numbers in an immunized host. This possibility was examined in two experiments.

The first experiment involved two groups of mice: one, a subgroup of a larger group that had been infected 12 wk previously with the standard inoculum of tubercle bacilli ( $6.2 \times 10^6$ ) intravenously and had received the two drugs thereafter according to the standard procedure; the other, a group of litter mates that had received no previous infection or drugs. No tubercle bacilli could be cultured from the lungs and spleen of a sample of 10 mice from the previously treated group when examined at the 12 wk point. The mice of both groups were then infected intravenously with the H37Rv strain of tubercle bacilli in a small inoculum (approximately 60 bacilli or 0.2 ml of a  $10^{-5}$  dilution of a 7 day culture in Tween-albumin medium). The microbial populations in lungs and spleen during the ensuing days were determined and are presented in Fig. 5.

As may be seen, in both the lungs and spleen during the first 10 days of infection, the populations rose to an identical census in both the control animals and in those possibly immunized by virtue of antecedent infection with large numbers of tubercle bacilli. In the last named animals, however, from this point on, the populations of tubercle bacilli failed to rise significantly. By contrast, in the control animals, the populations continued to rise so that the microbial census increased thereafter by more than a factor of ten in the spleens and in the lungs. This stabilization of the census at a lower level in the animals with antecedent infection is interpreted as evidence of acquired resistance. The delay, in this case 10 days, before any evidence of immunity could be detected after rechallenge, is characteristic of acquired resistance in experimental tuberculous infection.

In the immunized animals, rechallenge with a population of 60 units of tubercle bacilli was followed within 24 hr by the appearance of an easily detectable and steadily rising census of tubercle bacilli. It is presumed that this

detectable population represented descendants of the rechallenge bacilli rather than persists from the antecedent infection because: immediately prior to rechallenge, a sample subgroup revealed no culturable bacilli; and evocation of the artificially sterilized infection at this point in time has not been accomplished even with the administration of the large doses of cortisone.

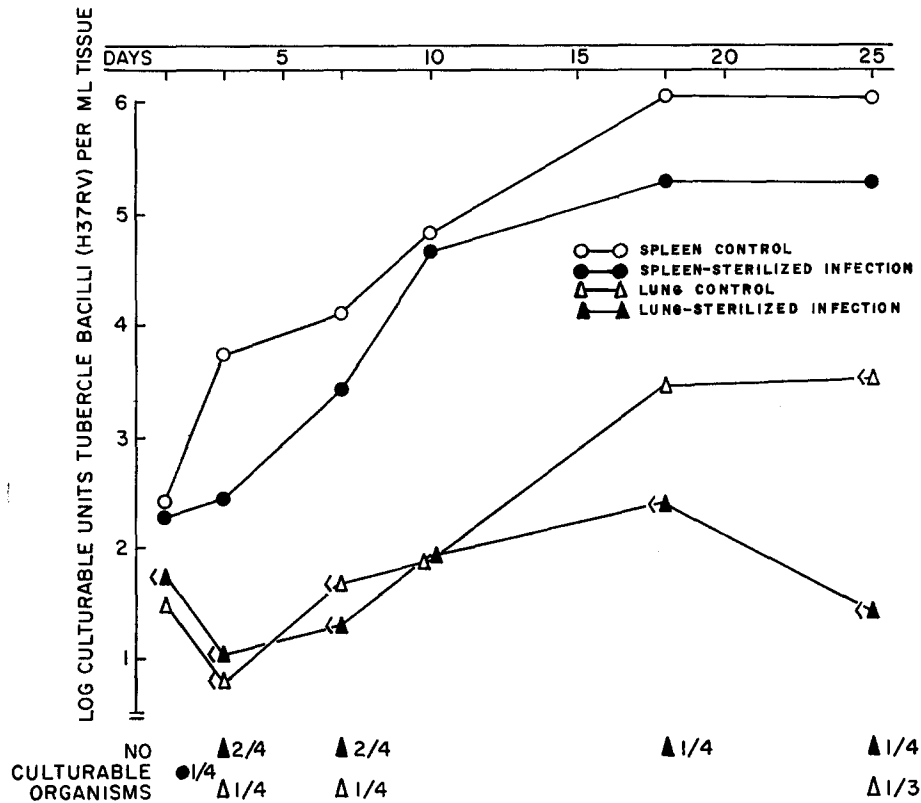


FIG. 5 The behavior of populations of tubercle bacilli in the lungs and spleens of mice rechallenged with a small inoculum ( $6.0 \times 10^1$ ) after a large population had been rendered sterile by the standard 12 wk regimen of pyrazinamide and isoniazid started within 20 min of infection.

The second experiment was similar to the first, except that the initial infection had been allowed to progress for 21 days before the 12 wk period of pyrazinamide-isoniazid administration was started. On the completion of drug administration, a control group and a group with antecedent infection received a challenge of 15 units of tubercle bacilli. Starting 24 hr later and for the 1st wk thereafter, populations of tubercle bacilli were readily detectable and increased identically in the spleens of both groups of animals (Fig. 6). From that point on, in the mice with the antecedent infection, the splenic census of tubercle bacilli



rose by a factor of ten and then fell. By contrast, in the control mice, the splenic census rose by more than a factor of one hundred and stabilized at this higher level.

The extent of acquired immunity, as reflected by the population differences, was greater in this second experiment. This presumably reflects the fact that in this experiment the initial infection had progressed for 21 days before drug

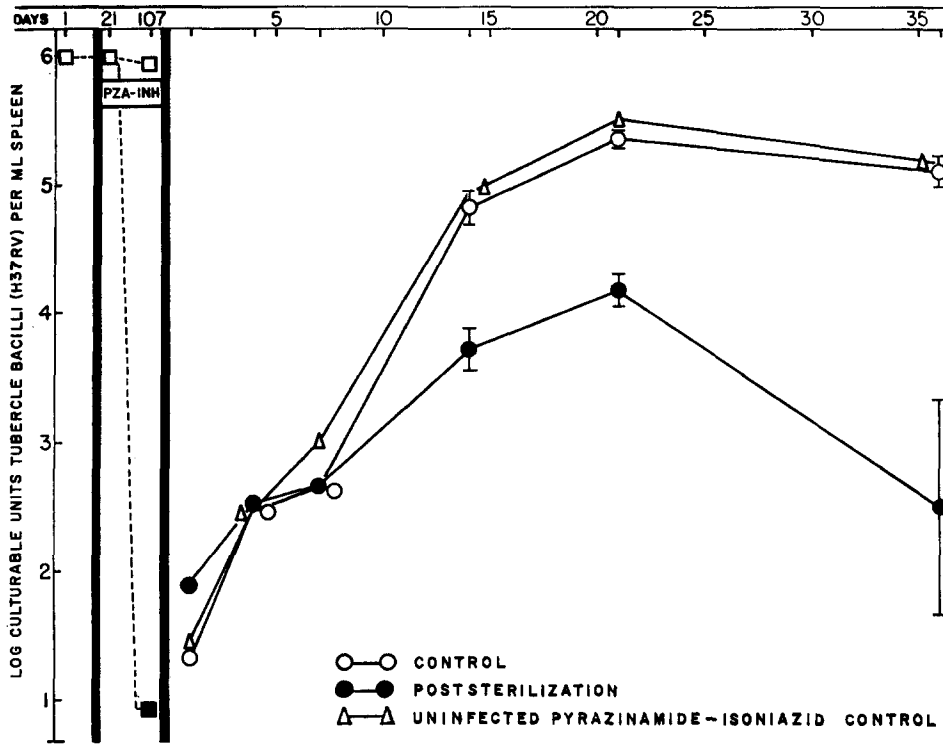


FIG. 6. The behavior of populations of tubercle bacilli in the spleens of mice rechallenged with a small inoculum ( $1.5 \times 10^3$ ) after a large population ( $10^6$ ) had been rendered sterile by the standard 12 wk regimen of pyrazinamide and isoniazid started 21 days after original infection.

administration, whereas in the first experiment the inoculum had been subjected to drug influence immediately after infection. In the second experiment, an additional control group that had not been infected but had received the two drugs for the 12 wk period, received the small inoculum of tubercle bacilli at the time of rechallenge of the previously infected animals. The behavior of the tubercle bacilli introduced into these previously treated mice was identical with that observed in the control group that had received no previous treatment.

This question of a possible role of acquired resistance interfering with the detection of bacilli in the posttreatment period was also studied with the use of BCG. The standard 12 wk regimen of pyrazinamide and isoniazid was administered, starting from the day of infection, to two groups of infected animals, one of which had been vaccinated with BCG 4 wk previously. At the end of the 12 wk period, there were no culturable tubercle bacilli in the lungs or spleens of the sample of animals sacrificed from either the vaccinated or the nonvaccinated drug-treated groups. 12 wk posttherapy, tubercle bacilli could be cultured from the spleens of 2 of the 15 vaccinated animals and 5 of the 15 nonvaccinated animals.

#### *Cross-Infection rather than Microbial Revival*

Two studies were made to explore the possibility that what was interpreted as microbial revival in the posttreatment period actually represented accidental reinfection with tubercle bacilli. In the first study, a group of 20 uninfected mice was housed in cages in immediate contact with the cages of mice that had been infected with tubercle bacilli by the intravenous route and were involved in some of the year-long experiments of the present studies. Sample groups of the uninfected mice were sacrificed periodically throughout a 12 wk period and the lungs and spleens were cultured for tubercle bacilli. No tubercle bacilli were found. In the second study, a group of 15 mice were infected and treated according to the standard model. At the 12 wk observation point, when the sample group revealed no culturable tubercle bacilli, all of the mice were removed to quarters in a different building in which no studies with any microbe were being conducted. The incidence of culturable tubercle bacilli in the lungs and spleens of these separately housed mice was the same at the 4, 8, and 12 wk posttreatment observation points as in the organs of similarly infected and manipulated mice maintained in the standard experimental conditions.

#### *Capacity of Revived Strains to Multiply in Vivo*

At the time of first detection in the posttreatment year, the census of tubercle bacilli varied over a wide range in both lungs and spleens. In the lungs from the 3rd to the 6th posttreatment months, the median census was  $10^3$ , a value comparable with that seen in nonimmunized animals 3 wk after infection with 60 culturable units of tubercle bacilli (Fig. 5). The median census in the spleens when first detected in this same period was  $10^4$ , a value that showed no significant change throughout the remainder of the year. The populations in the lungs in the second half of the year were somewhat higher when first detected but the variation was wide,  $10^1$  to  $10^8$ , with a median of  $10^4$ .

No systematic study was made of the colonial morphology and growth rate in vitro of every one of these several hundred "revived" strains of tubercle bacilli recovered from splenic or pulmonary tissue. When first isolated, however,

each culture was carefully inspected and all were subcultured. Many were also tested repeatedly for drug susceptibility in vitro. From these procedures, which were conducted routinely, the general statement can be made that the tubercle bacilli isolated as revived strains showed no unusual colonial morphology or incapacity to multiply in vitro, as compared with the parent strain H37Rv.

The capacity to multiply and to produce lesions was studied for three revived strains chosen at random. The circumstances of their first isolation and the experimental procedures are as follows:

All 3 strains were isolated from 74 mice that had been maintained for 9 to 12 months after completion of the 12 wk regimen of pyrazinamide and isoniazid. One strain was first isolated in high census ( $1.9 \times 10^8$ ) 1 year after completion of therapy. Another was isolated at the same time in the same experiment, but was present in a low census ( $7.5 \times 10^4$ ) when first cultured. The third strain was from a different experiment and was isolated in a census of  $8.6 \times 10^6$ , 9 months after completion of the drug administration.

These three strains and the stock strain H37Rv were each injected into different groups of mice by the procedures outlined in Materials and Methods. Sample subgroups of 4 or 5 mice from each of the four main groups were sacrificed at 24 hr and at 1, 3, and 9, wk thereafter. The census of tubercle bacilli in the spleens and lungs was determined at the time of sacrifice. These data are presented in Fig. 7.

As may be seen in fig. 7 with all 4 strains of tubercle bacilli, the populations in the spleens and lungs of the mice were roughly comparable 24 hr after infection. The 3 revived strains increased in census thereafter in a pattern identical with that shown by the stock H37Rv strain of tubercle bacilli. At the completion of the 9 wk experiment, small macroscopic lesions were present in the lungs of all animals irrespective of the strain of tubercle bacilli used for infection.

#### *Specificity of Isoniazid as the Companion Drug*

Isoniazid has been the drug used with pyrazinamide in all of the present studies and in virtually all of those previously reported or relating to this "disappearance" of the tubercle bacilli from the tissues. Nevertheless, it was reported previously (4) that the phenomenon could also be produced when the pyrazinamide was accompanied by such diverse substances as streptomycin, paraaminosalicylic acid, and oxytetracycline. With the present studies so largely focused on the detection procedures, this question of whether substances other than isoniazid could be substituted as companion drugs, was reexamined. Contrary to the previous report (4), when the more sensitive culturing techniques were used, complete disappearance of the populations of tubercle bacilli was not observed when streptomycin, oxytetracycline, or paraaminosalicylic acid were substituted for isoniazid as companion drugs for the pyrazinamide. Culturable tubercle bacilli were present in the spleens of: 1 of 8 animals in the pyrazinamide-streptomycin group; 2 of 8 animals in the pyrazinamide-para-

aminosalicylic acid (PAS) group; and, in 4 of 8 animals that had received pyrazinamide and oxytetracycline. Penicillin 1.5 mg daily by the intramuscular route was also ineffective as an isoniazid substitute when administered with pyrazinamide for the standard 12 wk period.

The acquired resistance produced by BCG as a possible substitute for the isoniazid was also studied. The BCG was administered 4 wk prior to the challenge with human tubercle bacilli and the start of pyrazinamide administration. At the 12 wk observation point, culturable tubercle bacilli were

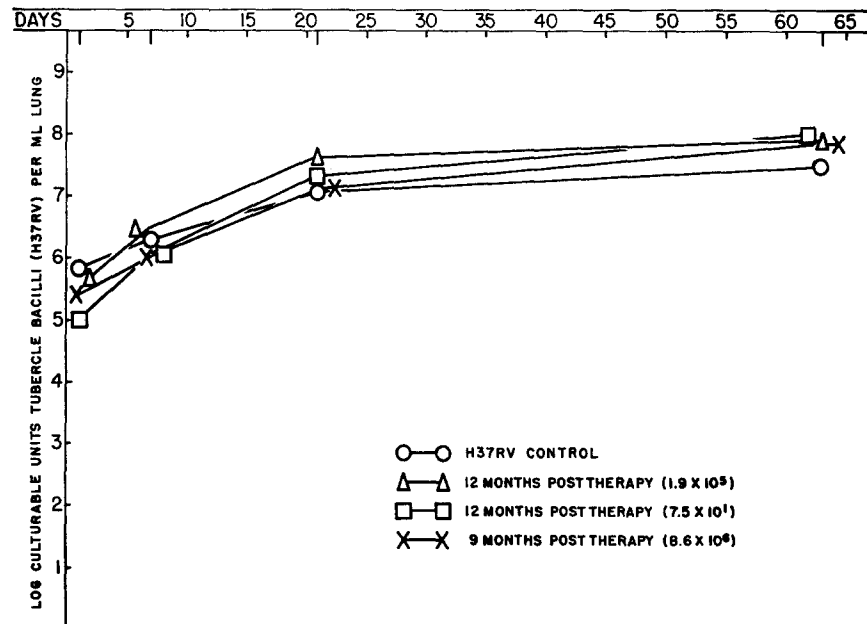


FIG. 7. A comparison of the capacity of three revired strains of tubercle bacilli and the standard strain H37Rv to multiply in the mouse lung. Inoculated intravenously with 0.2 ml of  $10^{-1}$  dilution of 7 day culture in liquid medium.

present in both the lungs and spleens of all of the vaccinated animals that had also received the pyrazinamide. From studies of suitable control groups, it was clear that the vaccination had resulted in acquired resistance and that it was highly unlikely that the bacilli cultured after 12 wk of pyrazinamide alone represented survivors from the vaccine rather than of the challenge inoculum.

#### DISCUSSION

From the above observations, it appears that the phenomenon whereby large populations of tubercle bacilli "vanish" from the tissues of mice only to

revive months later, occurs generally throughout each group of animals subjected to the experimental procedure. The phenomenon does not reflect the eradication of the bacilli in a significant portion of the animals. The evidence for this conclusion is of two sorts: the high percentage of microbial revival in the year following drug administration, with the normal sigmoid character of the revival curve; and the virtual uniformity with which microbial revival was evoked by the administration of large doses of cortisone during the 3rd or 4th posttreatment month. The cardinal characteristic of the tubercle bacilli while "vanished" is that they are sterile. By this is meant that they are incapable of having detectable progeny in the tissues of their host; in the tissues of a host to which they have been transplanted; or in a wide variety of cell-free media and cell cultures *in vitro*.

Once induced, the stability of the state in which the bacilli remain sterile is noteworthy. This may be seen in the consistent failure to find any culturable bacilli 1 month after completion of treatment, even after the daily administration of 1.0 mg of cortisone and in the failure to evoke culturable bacilli in the major portion of the animals by the administration of cortisone during the 2nd posttreatment month.

In the natural revival, the difference between the appearance of culturable tubercle bacilli in only one-third of the animals at 3 months, and their appearance in two-thirds of the animals at 9 months, indicates that the nonculturable state may exist for months. This difference in the incidence of revival at 9 or 12 months and the almost uniform revival induced by cortisone in the 3rd or 4th month, suggests that in some animals, perhaps one-third, there are sterile but potentially revivable tubercle bacilli present at 4 months that fail to survive or at least to revive, thereafter. The continued absence of these bacilli took place in immunized hosts. In the "blind transfer" experiments, however, the recipient hosts had not been immunized, yet no culturable tubercle bacilli appeared in their tissues following their inoculation with splenic homogenates that presumably contained sterilized but potentially revivable bacilli. These two sets of observations suggest: that sterile bacilli in the earliest stages of revival are more vulnerable than orthodox bacilli to some factor in the native resistance of the mouse; and that some bacillary process requiring time must take place before this vulnerability is lost. The increased percentage of revival effected by the near lethal dosage of cortisone in the 3rd and 4th posttreatment months (but not the 1st month) is in keeping with this hypothesis. Thus the ultimate fate of the populations of tubercle bacilli in a substantial portion of the animals may be still undecided as late as three months after completion of their drug exposure. In these animals, which appear to represent about one-third, the populations of tubercle bacilli obviously still have the *capacity* to revive as revealed by the cortisone but they do not in fact revive within the ensuing 9 months.

To what extent acquired resistance might exert a continued influence on the sterile bacilli cannot be stated. From the rechallenge experiments, however, it is clear that the acquired resistance that developed in the animals of the standard model did not interfere with the culturability of very small numbers of orthodox tubercle bacilli known to be capable of multiplication.

All attempts to find fertile bacilli by increasing the sensitivity of the detection techniques were failures. Whether the typical microbial forms detected by intensive microscopy of the tissue homogenates represent the living bacilli which ultimately revive, cannot be determined from the present evidence. Until this point is established, it is not correct to designate the "vanished" infection as *latent* as was done in previous publications (2, 3). For, the persisters that ultimately revive may either be these visible microbial forms or have arisen from them by using some surviving portion as a template. The continued uncertainty on the point is by no means unreasonable. For example, in connection with the present studies, acid-fast microbial forms were readily visualized in the tissues of both treated and untreated mice 12 wk after intravenous inoculation with a culture of tubercle bacilli that had been subjected to a temperature of 80 degrees Centigrade for 1 hr. Cultures of both the inoculum and the tissues obtained 5 and 6 months later yielded no tubercle bacilli. Yet microbial revival was observed in one of 3 animals sacrificed 4 months after the inoculation with the "heat-killed" tubercle bacilli. Thus, the possibility that the forms visible at 12 wk represented "sterile" rather than "dead" bacilli was not excluded. However, Steenken (15) has reported that tubercle bacilli that have been "killed", i.e. rendered sterile by high temperatures, may nevertheless persist with characteristic morphology for many months in the tissues of guinea pigs. Rees and Hart (16) had no difficulty demonstrating acid-fast microbial forms in mouse lungs after the administration of pyrazinamide and isoniazid for 115 days at which time there were no culturable tubercle bacilli. In their experiments, however, the infection with tubercle bacilli had been allowed to progress for 60 days before drug administration was started. This is in contrast to the present studies with the 12 wk model in which both infection and drug administration are started on the same day. Steenken, Wolinsky, Smith, and Montalbino (17) have reported studies of pyrazinamide and isoniazid, and an isoniazid derivative, in tuberculous guinea pigs. When drug administration was started 6 days after infection and continued for 123 days (17 wk), they found culturable tubercle bacilli in the spleen of one of 13 animals sacrificed. No data on microscopy are reported.

Attempts are being made to clarify this issue of the relation of the revived bacilli to the visible forms, by electron microscopy of tubercle bacilli that have survived pyrazinamide-isoniazid exposure. The number of such bacilli that become available are so few, however, that thus far, these attempts have been defeated by technical problems.

Irrespective of whether the persisting bacilli in the present study are visible or invisible, it is clear that they have undergone significant alteration in that the populations have been rendered uniformly sterile. Observations are presented in the paper that follows (6) that serve to define a number of the characteristics of this sterile state.

#### SUMMARY

A previously reported form of microbial persistence whereby large populations of tubercle bacilli can be made to "vanish" uniformly from the tissues of mice has been shown to occur generally throughout each group of animals subjected to the experimental procedure; it does not reflect the eradication of the bacilli in the majority of animals with their persistence and ultimate revival in only a minority. The one demonstrable alteration of the tubercle bacilli while "vanished" is that they are sterile. Thus, they are undetectable by cell-free culture, tissue culture, and blind animal passage, i.e. by any method based on microbial multiplication. Whether they have also undergone alteration in morphology and persist in some unconventional form cannot be stated. Acid-fast forms similar to tubercle bacilli can be detected in small numbers by intensified microscopic search of tissue homogenates but the relationship of these forms to the sterile bacilli that ultimately revive is unclear. Thus, the persisting tubercle bacilli are more correctly designated as being in a "sterile state" than one of true latency. The *uniform* induction of the sterile state is a specific phenomenon requiring the participation of both the nicotinamide derivative, pyrazinamide, and isoniazid. Once assumed, this sterile state is relatively stable and the time required for revival of the tubercle bacilli in the spleens in one-half the animals is seven months. This process can be speeded up by the administration of large doses of cortisone in the third or fourth month after sterilization but revival is not significantly affected by the administration of cortisone earlier.

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